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Bioassay-Guided Investigation of Two *Monarda* Essential Oils as Repellents of Yellow Fever Mosquito *Aedes aegypti*

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ABSTRACT: As part of an ongoing research program to identify active mosquito repellents, *Monarda bradburiana* Beck and *Monarda fistulosa* L. essential oils showed good repellent activity with minimum effective dosages (MED) of 0.055 ± 0.036 and 0.078 ± 0.027 mg/cm^2, respectively, compared to reference standard *N*,*N*-diethyl-3-methylbenzamide (DEET) (0.039 ± 0.014 mg/cm^2). Systematic bioassay-guided fractionation of essential oils of both *Monarda* species was performed to identify the active repellent compounds, and isolated pure compounds were individually tested for repellency. Of the isolated compounds, carvacrol, thymol, eugenol, and carvacrol methyl ether were found to be the repellent compounds with MEDs in the range of 0.013−0.063 mg/cm^2. Active repellent compounds were also tested for larvicidal activity against 1-day-old *Aedes aegypti* larvae. Thymol was the best larvicide among the tested individual compounds (LD_{50} of 13.9 ppm). None of the individual compounds showed cytotoxicity against mammalian cells; however, the essential oils were toxic to all cell lines.

KEYWORDS: *Monarda bradburiana, Monarda fistulosa, mosquito repellent, carvacrol, thymol, eugenol, carvacrol methyl ether, (R)-(-)-1-octen-3-ol, cytotoxicity*

INTRODUCTION

Vector-borne diseases are a major public health problem, and new vector-borne threats continue to emerge in regions where they had not previously been an issue. *Aedes aegypti* L. is a global vector of dengue fever and yellow fever, both of which can cause severe human morbidity and mortality. In the United States, dengue infections are generally seen in travelers or migrant workers; however, autochthonous dengue fever was recorded in Hawaii, in 2001; Brownsville, TX, in 2005; and southern Florida, in 2009−2011.[1,2] Development of new insecticides to control mosquitoes and the use of repellents may reduce or prevent mosquito borne diseases.[3] The compound DEET (*N*,*N*-diethyl-3-methylbenzamide) is the most commonly used arthropod repellent on the market.[4] There are other insect repellents, such as picaridin [KBR 3023 or 2-(2-hydroxyethyl-1-piperidincarboxylic acid 1-methylpropyl ester)], IR 3535 (3-[butyl-*N*-acetyl]aminopropionic acid ethyl ester), SS220 [(1*S*,2*S*)-methylpiperidinyl-3-cyclohexen-1-carboxamide], and PMD (*p*-menthane-3,8-diol), that have demonstrated repellency similar to that of DEET.[5] There exist cases in which high levels of DEET exposure have caused neurotoxicity, dermatitis, and allergic reactions.[6,7] DEET can dissolve or damage plastics and harm some synthetic clothes. The development of tolerance to DEET in mosquitoes has also been reported.[8] As a result of these issues, there has been a continued research effort to identify new mosquito repellents as alternatives to DEET. Plants have been major sources for the discovery of novel natural insecticides and repellents.[9] On the basis of our preliminary screening results from medicinal and aromatic plants, essential oils from *Monarda bradburiana* Beck and *Monarda fistulosa* L. showed good repellent activity and thus were chosen for further investigation.

The genus *Monarda* L. (Lamiaceae) is represented by 15 species native to the United States.[10] *M. bradburiana* (eastern bee balm, white horsemint) leaves have been used to make tea for fevers, upset stomach, and digestive gas; as a cold and cough remedy; and as a pleasant beverage.[11] *M. fistulosa* (wild bergamot) has been used medicinally by Native American groups for treating wounds, heart problems, colds and flu, stomach pain, nose bleeding, fever, gas, headache, insomnia, skin problems, and infant convulsions.[12] Native Americans also used the plant to make perfumes, utilized the leaves as incense, and sprinkled pulverized leaves on meats to repel insects.[12] Duke indicates that *M. fistulosa* has also been used to cure cancer, melanoma, and worms.[13] Zhan-Guo et al. found that *Monarda citriodora* essential oil has strong antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus*

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14. ABSTRACT

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Table 1. Chemical Composition of the Essential Oils of *Monarda bradburiana* (Mb) and *M. fistulosa* (Mf)

no.	RRI[a]	compd	Mb (%)[b]	Mf (%)[b]	identification[c]
1	1032	α-pinene	0.7	0.8	t_R, MS
2	1035	α-thujene	2.0	2.1	MS
3	1076	camphene	0.1		t_R, MS
4	1118	β-pinene	0.2	0.2	t_R, MS
5	1159	δ-3-carene	0.1		MS
6	1188	α-terpinene	1.3	2.5	t_R, MS
7	1203	limonene	0.8	0.9	t_R, MS
8	1218	β-phellandrene	0.3	0.4	t_R, MS
9	1255	γ-terpinene	0.5	1.1	t_R, MS
10	1280	*p*-cymene	17.6	35.4	t_R, MS
11	1290	terpinolene	0.1		t_R, MS
12	1393	3-octanol	0.1		t_R, MS
13	1452	α,*p*-dimethylstyrene	0.1		MS
14	1452	1-octen-3-ol	6.7	10.3	t_R, MS
15	1479	furfural	0.1		MS
16	1497	α-copaene	<0.1		t_R, MS
17	1553	linalool	0.3	0.3	t_R, MS
18	1611	terpinen-4-ol	1.3	1.5	t_R, MS
19	1612	β-caryophyllene	1.7	2.1	t_R, MS
20	1614	carvacrol methyl ether		0.2	t_R, MS
21	1695	(*E*)-β-farnesene	1.4		MS
22	1704	γ-muurolene	0.2	0.3	MS
23	1706	α-terpineol	0.2	0.2	t_R, MS
24	1719	borneol	0.2	0.2	t_R, MS
25	1726	germacrene D	0.1	0.3	MS
26	1740	valencene		0.2	MS
27	1740	α-muurolene	0.1		MS
28	1744	α-selinene	0.1		MS
29	1773	δ-cadinene	0.2	0.3	MS
30	1776	γ-cadinene	0.1	0.2	MS
31	1802	cumin aldehyde	<0.1	0.1	t_R, MS
32	1849	calamenene	<0.1	0.1	MS
33	1864	*p*-cymen-8-ol	0.3	0.5	t_R, MS
34	1940	4-isopropyl salicylaldehyde		0.1	MS
35	1941	α-calacorene	<0.1		MS
36	2008	caryophyllene oxide	0.2	0.2	t_R, MS
37	2113	cumin alcohol		0.1	t_R, MS
38	2186	eugenol	0.4		t_R, MS
39	2198	thymol	57.7	0.2	t_R, MS
40	2239	carvacrol	4.6	39.1	t_R, MS
		total	99.8	99.1	

[a]RRI: relative retention indices. [b]Percent calculated from FID relative peak area data. [c]Identification method: t_R, identification based on the retention times (t_R) of genuine compounds on the HP Innowax column; MS, identified was performed on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

albus.[14] Zhilyakova et al. reported that *M. fistulosa* essential oil exhibited antibacterial activity against Gram-positive and -negative microorganisms and also stimulated anti-inflammatory activity in a mixture with hydrocortisone and vitamin B6.[15] In another recent study, an 80% acetone extract of *M. punctata* showed an inhibitory effect on lipase activity in isolated mouse plasma in vitro with an IC_{50} value of 2.0 mg/mL. Carvacrol, the major compound from *M. punctata* extract, had potential lipase activity, with an IC_{50} value of 4.07 mM, and suppressed the postprandial elevation of blood triacylglycerol concentrations in mice in vivo; however, five other monoterpene glycosides that were isolated did not show the mouse lipase inhibitory activity.[16] Repellent studies of two *Monarda* species in Oklahoma showed *Monarda punctata* and *M. citriodora* essential oils (with most abundant compounds being thymol and carvacrol) to be repellent to adult mosquitoes.[17,18] Both *Monarda* essential oils were further evaluated against *Drosophila melanogaster*, and the oils (with dominant compounds being thymol and carvacrol) were reported as repellent at low concentrations.[19] Since *Monarda* essential oils are known to be repellent to mosquitoes, this present study investigates the repellent activity of the essential oils of *M. bradburiana* and *M. fistulosa* against *Ae. aegypti* L. Systematic bioassay-guided fractionation of *M. bradburiana* and *M. fistulosa* essential oils was performed to identify active repellent compounds against *Ae. aegypti*. The isolated pure compounds were individually tested in mosquito repellent bioassays, and active repellent compounds were also evaluated for their larvicidal activity

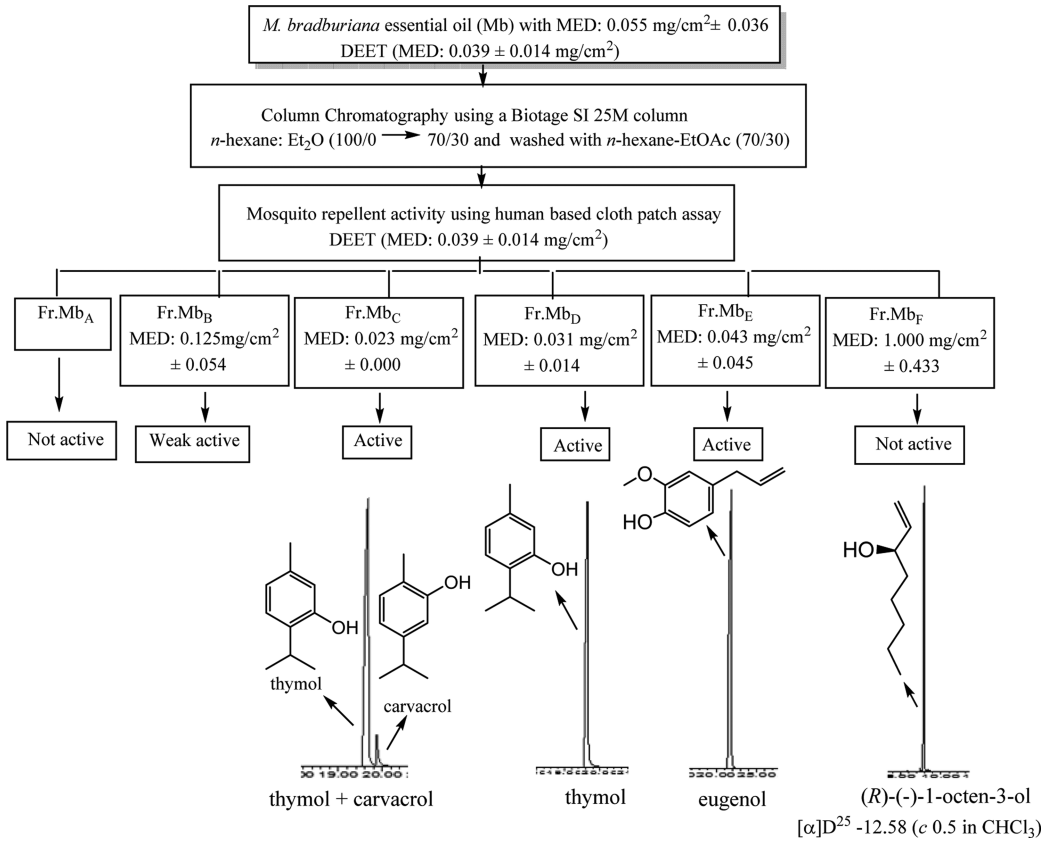

Figure 1. A flowchart illustrating procedures for testing *M. bradburiana* essential oil (Mb) and its mosquito repellent activity. Repellency was assessed by measuring the minimum effective dosage (MED) for essential oils or compounds and DEET was used as a positive control.

against 1-day-old *Ae. aegypti* and cytotoxicity against mammalian cells.

MATERIALS AND METHODS

Chemicals. Carvacrol (CAS# 499-75-2), thymol (CAS# 89-83-8), and eugenol (CAS# 97-53-0) were purchased from Sigma-Aldrich Co. (St. Louis, MO), and carvacrol methyl ether (CAS# 6379-73-3) was purchased from Waterstone Technology Inc. (Carmel, IN). Authentic (*R*)-(−)- and (*S*)-(+)-1-octen-3-ol were supplied by Bedoukian Research, Inc. (Danbury, CT).

Plant Material. Plants used in this study were propagated from seeds obtained from Jelitto Perennial Seeds (Louisville, KY) and grown in field rows in a sandy loam soil at the South Mississippi Branch Experiment Station in Poplarville, MS (30°50′26″N, long. 89°32′46″W; USDA hardiness zone 8b). Aerial parts of *M. bradburiana* and *M. fistulosa* were harvested and air-dried inside an air-conditioned building. Voucher specimens have been deposited at the National Center for Natural Products Research (NCNPR# 13149 and 13150, respectively), University of Mississippi (University, MS).

Essential Oils. Aerial parts of *M. bradburiana* and *M. fistulosa* were separately subjected to hydrodistillation for 3 h using a Clevenger-type apparatus to produce the oils. Light yellow oil was obtained from the *M. bradburiana* with 0.14% (v/w) yield. Yellow oil was obtained from *M. fistulosa* with 0.43% (v/w) yield.

Gas Chromatography and Gas Chromatography Mass Spectrometry Analysis for Essential Oils. *M. bradburiana* (Mb) and *M. fistulosa* (Mf) essential oils were analyzed by gas chromatography (GC) with a flame ionization detector (FID) and gas chromatography−mass spectrometry (GC−MS) using an Agilent GC−mass selective detector (MSD) system. The GC−MS analyses were done with an Agilent 5975 GC-MSD system. An Innowax fused silica capillary (FSC) column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium as the carrier gas (0.8 mL/min). The oven temperature was kept at 60 °C for 10 min, programmed to 220 °C at a rate of 4 °C/min, maintained constant at 220 °C for 10 min, and finally programmed to 240 °C at a rate of 1 °C/min. The injector temperature was set at 250 °C. The split flow was adjusted to 50:1. Mass spectra were recorded at 70 eV with the mass range *m*/*z* 35−450.

The GC analyses were performed using an Agilent 6890N GC system. FID detector temperature was set to 300 °C, and the same operational conditions were applied to a duplicate of the same column used in GC−MS analyses. Simultaneous autoinjection was done to obtain equivalent retention times. Relative percentages of the separated compounds were calculated from integration of the peak areas in the GC−FID chromatograms (Table 1).

Identification of essential oil components was accomplished by comparison of retention times with authentic samples or by comparison of their relative retention index (RRI) to a series of *n*-alkanes.[20] Computer matching with commercial mass spectral libraries (Wiley GC/MS Library, MassFinder 3 Library) and the in-house "Baser Library of Essential Oil Constituents", which includes over 3200 genuine compounds with MS and retention data from pure standard compounds and components of known oils as well as MS literature data, were also used for identification.[21−24]

Bioassay-Guided Fractionation and Purification of the Bioactive Compounds. Both *Monarda* essential oils were evaluated for their mosquito repellent activity against *Ae. aegypti*. A systematic bioassay-guided fractionation was carried out using cloth patch repellency bioassays to isolate and identify compounds with mosquito repellent activity and without any cytotoxicity.

M. bradburiana (500 mg) and *M. fistulosa* (500 mg) essential oils were fractionated separately by high-performance flash chromatography (HPFC) using a Biotage SI instrument with a 25 M silica column (flow rate 5.0 mL/min) and eluted with *n*-hexane (100%, 210 mL), *n*-hexane−Et_2O mixtures (99/1 to 98/2, 210 mL; 98/2 to 93/7, 180 mL; 97/3 to 96/4, 180 mL; 96/4, 120 mL; 96/4 to 95/5, 120 mL; 95/5 to 90:10, 120 mL, 90/10 to 80/20, 90 mL, 80/20 to 70/30, 90

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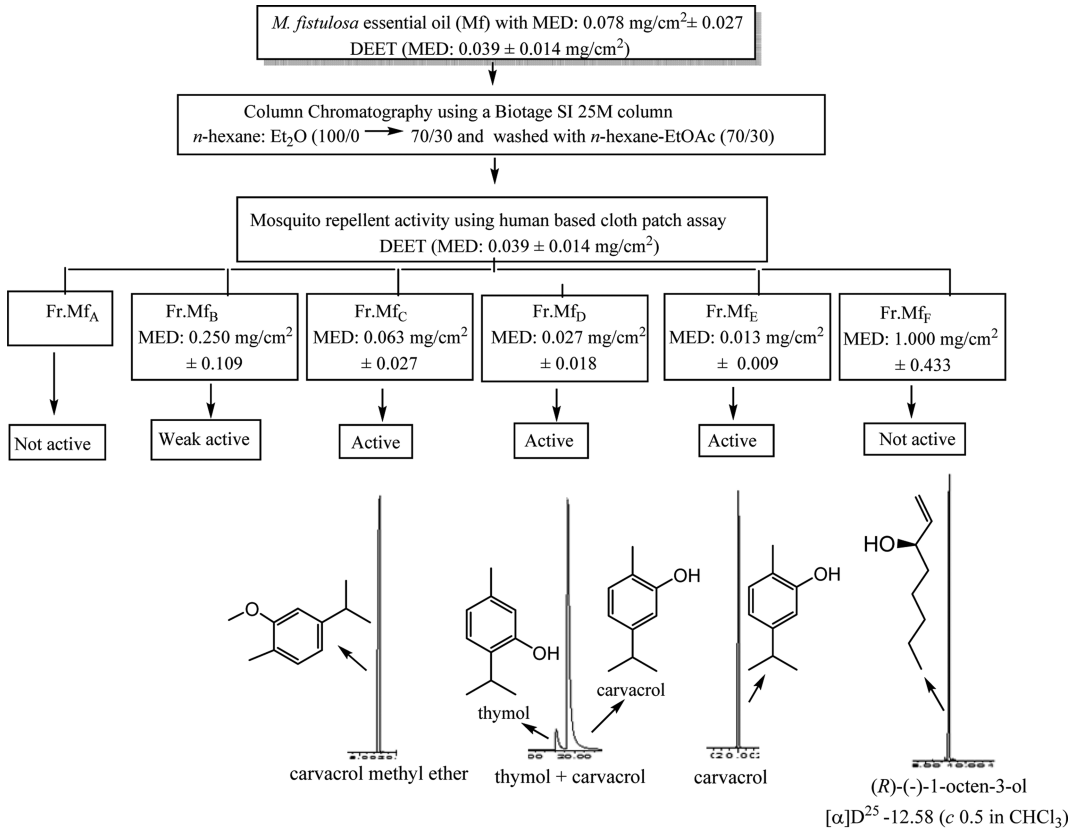

Figure 2. A flowchart illustrating procedures for testing *M. fistulosa* essential oil (Mf) and its mosquito repellent activity against *Ae. aegypti*. Repellency was assessed by measuring the minimum effective dosage (MED) for essential oils or compounds and DEET was used as a positive control.

mL) and finally washed with *n*-hexane−EtOAc (70/30, 240 mL). Portions of 3 mL volume were collected in 16 × 100 mm test tubes. Fractions with similar TLC profiles (*n*-hexane−Et_2O 95/5, 90/10, 85/15, 80/20, 70/30 v/v) were combined to give six fractions. Subfractions for *M. bradburiana* (Mb_{A-F}) were as follows: Mb_A, 100 mg (mixture); Mb_B, 30 mg (mixture); Mb_C, 90 mg (mixture of thymol and carvacrol); Mb_D, 220 mg (thymol); Mb_E, 10 mg (eugenol); and MB_F, 30 mg (1-octen-3-ol) (Figure 1). Subfractions for *M. fistulosa* (Mf_{A-F}) were as follows: Mf_A, 125 mg (mixture); Mf_B, 25 mg (mixture); Mf_C, 10 mg (carvacrol methyl ether); Mf_D, 50 mg (mixture of carvacrol and thymol); Mf_E, 250 mg (carvacrol); and Mf_F, 50 mg (1-octen-3-ol) (Figure 2). Both Mb_{A-F} and Mf_{A-F} subfractions were analyzed by GC−MS and all fractions were evaluated for mosquito repellent activity against *Ae. aegypti* (Figures 1 and 2). Since the isolated amounts of eugenol (10 mg) and carvacrol methyl ether (10 mg) were insufficient for the mosquito repellent activity bioassays, eugenol and carvacrol methyl ether were purchased for the bioassays.

Gas Chromatography−Mass Spectrometry Analysis for Subfractions and Isolated Compounds. Subfractions (Mb_{A-F} and Mf_{A-F}) and isolated pure compounds (thymol, carvacrol, eugenol, carvacrol methyl ether, 1-octen-3-ol) from both *Monarda* essential oils were compared with standards purchased from commercial sources and analyzed using an HP 5890 series gas chromatography linked to an HP 5970 mass spectrometer system equipped with an HP automatic injector and DB-1 capillary column (20 m × 0.18 mm with 0.25 μm film thicknesses). Helium (1 mL/min) was used as carrier gas. The GC oven temperature was held at 70 °C for 3 min, ramped to 220 °C at a rate of 4 °C/min, and then held constant at 220 °C for 5 min, followed by a final increase to 240 °C at a rate of 1 °C/min. The scanned mass range was from *m*/*z* 40 to 550 and the ion source was operated in electron ionization (EI) mode with an electron energy set to 70 eV.

Chiral Separation of 1-Octen-3-ol. Enantiomeric separation of 1-octen-3-ol isolated from both *Monarda* oils was effected using an HP 5890 series gas chromatography linked to an HP 5970 mass spectrometer system on an Rt-βDEXsa column [30 m × 0.25 mm i.d. with a 0.25 μm layer of 2,3-diacetoxy-6-*O*-(*tert*-butyldimethylsilyl)-β-cyclodextrin; Restek Chromatography Products, Bellefonte, PA]. The chiral column was maintained at 60 °C for 5 min and then programmed to 60 °C at a rate of 1 °C/min. The injector temperature was set at 250 °C. The split flow was adjusted to 10:1. Mass spectra were recorded at 70 eV with the mass range *m*/*z* 40−500. The (*R*)-(−)-1-octen-3-ol isolated from both *Monarda* oils was compared with authentic (*R*)-(−)- and (*S*)-(+)-1-octen-3-ol (Bedoukian Research, Inc., Danbury, CT) to confirm their enantiomeric identity (Figure 3). The optical rotation measurements of 1-octen-3-ol isolated from *Monarda* oils were also completed on a Rudolph Research Analytical digital polarimeter in $CHCl_3$ at 25 °C.

In-Cage Mosquito Repellent Bioassay. Pupae of *Ae. aegypti* from the Center for Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville colony, were maintained in the laboratory at 28 ± 1 °C and 30−60% RH, and the resulting adult females aged 5−9 days were selected from the stock cages by a hand-draw box.[25] Repellency was determined as the MED, which is the minimum threshold surface concentration necessary to prevent mosquitoes from biting through the treated surface.[26] *Aedes aegypti* pupae were maintained in the laboratory until adult emergence. Approximately 500(±10%) mosquitoes were collected and loaded into a test cage (size of 45 cm ×37.5 cm × 35 cm) and held in the cage for 25(±2.5) min before initiating repellency assays.[27]

Test compounds were weighed in 2-dram vials and dissolved in 2 mL of ethanol. The initial mass of the chemical was measured so that when one-half was removed and a 50 cm^2 muslin cloth was added to the vial, the remaining 1 mL of solution would produce an initial concentration on the cloth of 1.5 mg/cm^2. Serial dilutions were then made such that the concentrations on the cloth for the remaining 1 mL solution were 1.5, 0.75, 0.375, 0.094, 0.047, 0.023, and 0.011 mg/cm^2. The vials were sealed and stored at −4 °C until testing (normally

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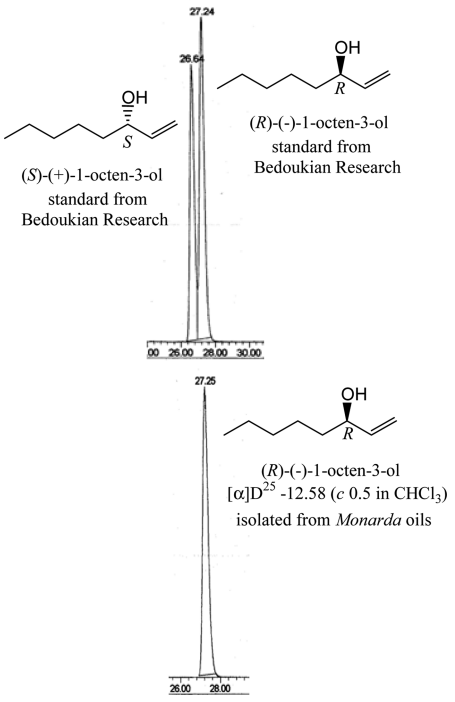

Figure 3. Enantiomeric separation of 1-octen-3-ol from *M. bradburiana* (Mb) and *M. fistulosa* (Mf) essential oils using an Rt-βDEXsa chiral column [2,3-diacetoxy-6-*O*-(*tert*-butyldimethylsilyl)-β-cyclodextrin].

<48 h). The cloth from the vial was removed and attached by staples onto two sections of card stock (5 cm × 2.5 cm). Approximately 1−2 in. of masking tape secured the cloth onto the card stock. The card and cloth assembly was placed on a drying rack for 3−5 min before the bioassay was carried out.

A single test consisted of covering the hand of a volunteer with a soft-embossed long cuff poly glove (Atlantis Products, Mankato, MN) and then with a second glove which was powder-free latex (Diamond Grip, Microflex Corp., Reno, NV). A knee-high stocking (Leggs everyday knee highs, Winston-Salem, NC) was then placed over the gloved hand and arm. A plastic sleeve constructed of polyvinyl was the final layer that was then affixed over the stocking-covered arm. The plastic sleeve was sealed around the arm by a Velcro strip. About half way between the wrist and elbow was a 4 × 8 cm opening used to assess mosquito landing and biting behavior. The opening permitted odors from the skin surface to emanate out and attract mosquitoes to this opening in the sleeve. During testing, this 32 cm^2 open area was covered with chemical-treated muslin cloth.

If zero to four bites were received through the treated cloth during the 1 min test period, the dosage of repellent on the cloth was considered to have "passed." A failing treatment received five bites out of 500 mosquitoes. An intermediate dosage (e.g., 0.187 mg/cm^2) was tested first. Depending on whether this concentration passed or failed, higher or lower treatment concentrations were evaluated with all subjects until each had pinpointed their individual concentration that produced the 1% (five bites) failure point. If the 1.5 mg/cm^2 (or highest concentration) on cloth was not efficacious (>5 bites in one minute), then the MED was noted as ineffective at the highest concentration tested. Because the mosquitoes show reduced behavioral activity upon repeated exposure to repellent and attractant odors from the arm, a limit of 10 successive tests was used, after which the caged mosquitoes were allowed a 15 min recovery period.

Two male volunteers participated in the studies of the MED of the oils. During a test, one or both volunteers wore a patch and tested each patch for 1 min intervals. Patches were rotated among the volunteers; thus, no patch was evaluated beyond 10 min after the 3 min drying period to avoid any bias that might result from evaporative loss of the treatment from the cloth during the duration of the test. All subjects provided written informed consent. The protocol was approved by the University of Florida Human Use Institutional Review Board-01 (Study # 636-2005). DEET (99.1% purity; *N,N*-diethyl-3-methylbenzamide) was obtained from Sigma Aldrich (St. Louis, MO) and used as a positive control. Ethanol was used as the negative control.

Larval Bioassays. Bioassays were conducted by using the bioassay system described by Pridgeon et al.[28] to determine the larvicidal activity of isolated compounds from *Monarda* species against *Ae. aegypti*. In brief, eggs were hatched under vacuum by placing a piece of a paper towel with eggs in a cup filled with 100 mL of deionized water containing a small quantity of larval diet (2% slurry of 2:1 alfalfa pellets and hog chow). Larvae were removed from vacuum and held overnight in the cup in a temperature-controlled chamber maintained at a temperature of 27 ± 2 °C and 70 ± 5% RH at a photoperiod regimen of 12:12 (L:D) h. Five 1-day-old first instar *Ae. aegypti larvae* were added to each well of 24-well plates placed on an illuminated light box by using a disposable 22.5-cm Pasteur pipet with a droplet of water. Fifty microliters of larval diet were added to each well by using a Finnpipette stepper (Thermo Fisher, Vantaa, Finland). All chemicals to be tested were diluted in dimethyl sulfoxide (DMSO). Eleven microliters of the test chemical was added to the labeled wells, and in control treatments 11 μL of DMSO alone was added. Each well had a total volume of 1.1 mL. After the treatment, the plates were swirled in clockwise, counterclockwise, front-to-back, and side-to-side motions five times to ensure even mixing of the chemicals. Larval mortality was recorded 24 and 48 h after treatment. Larvae that showed no movement in the well after manual disturbance of water by a pipet tip were recorded as dead. A series of dosages (four or five concentrations) were used in each treatment to get a range of mortality. Treatments were replicated 15 times in each extract.

Cytotoxic Activity. The two *Monarda* essential oils and isolated pure compounds were tested for their cytotoxicity against mammalian cells. Four human cancer cell lines (SK-MEL, malignant, melanoma; KB, epidermal carcinoma, oral; BT-549, ductal carcinoma, breast; SK-OV3, ovary carcinoma) and two noncancerous cell lines (VERO, monkey kidney fibroblast; LLC-PK11, pig kidney epithelial cells) obtained from ATCC were selected for the assay. The assay was performed in 96-well tissue-culture-treated microplates. Cells (25 000 cells/well) were seeded to the wells of the plate and incubated for 24 h. Samples were added and again incubated for 48 h. The number of viable cells was determined using the supravital dye Neutral Red according to a modification of the previous procedure.[29] Briefly, cells were washed with saline followed by a 1.5 h incubation in a solution of neutral red. The cells were washed again to remove extracellular dye. A solution of acidified isopropanol was added to liberate the incorporated dye from viable cells and the absorbance was read at 540 nm. Percent cell viability was calculated and plotted against test concentrations. IC_{50} values (the concentration that caused a 50% reduction in cell viability) were obtained from dose−response curves.

RESULTS AND DISCUSSION

The water-distilled essential oils from aerial parts of *M. bradburiana* and *M. fistulosa* were characterized by GC−FID and GC−MS. The compounds identified from the essential oils along with their relative percentages are listed in Table 1. Thirty-six compounds, which accounted for 99.8% of the total oil, were identified in the oil of *M. bradburiana*. Twenty-eight compounds, which accounted for 99.1% of the total oil, were identified in the oil of *M. fistulosa.* The major compounds in the essential oils of *M. bradburiana* were thymol (58%), *p*-cymene (18%), 1-octen-3-ol (7%), and carvacrol (5%). In comparison, carvacrol (39%), *p*-cymene (35%), and 1-octen-3-ol (10%) were major components of the essential oil of *M. fistulosa*. Thus, *M. bradburiana* essential oil was rich in thymol and *M. fistulosa* essential oil was rich in carvacrol as the major constituent. The

Table 2. Toxicity of Isolated Repellent Compounds from *M. bradburiana* (Mb) and *M. fistulosa* (Mf) Essential Oils against 1-Day-Old Larvae of *Aedes aegypti* at 24 h post-treatment

compd	LD_{50} (95% CI) ppm	LD_{90} (95% CI) ppm	Wald χ^2	DF
thymol	13.9 (12.1−16.0)	31.1 (25.8−40.5)	79.5	48
carvacrol	20.13 (17.7−22.8)	38.9 (33.0−49.2)	79.8	48
eugenol	23.4 (20.2−27.2)	57.5 (46.9−76.3)	96.0	48
carvacrol methyl ether	40.6 (36.4−45.2)	84.1 (72.8−101.6)	131.2	73

composition of the *M. bradburiana* essential oil has been published here for the first time.

M. bradburiana and *M. fistulosa* essential oils were repellent at MEDs of 0.055 ± 0.036 and 0.078 ± 0.027 mg/cm^2, respectively, compared to an MED of 0.039 ± 0.014 mg/cm^2 for DEET (Figures 1 and 2). A systematic bioassay-guided fractionation of both *Monarda* essential oils was performed to identify the compounds that contribute to the repellency of the oil. Column chromatography of the *M. bradburiana* essential oil resulted in six fractions. The MEDs for each fraction were evaluated against *Ae. aegypti* (Figure 1). No repellent activity was observed in fraction A (rich in *p*-cymene). Since fraction B showed a weak repellency with an MED of 0.125 ± 0.054 mg/cm^2, we did not further examine the chemical composition of this fraction. Fraction C contained a mixture of 92.55% thymol + 7.45% carvacrol and exhibited potent repellency with an MED of 0.023 mg/cm^2 ± 0.000. In the next fraction (D), thymol was contained in pure form and the MED of this fraction was 0.031 ± 0.014 mg/cm^2. The next fraction (E) contained eugenol and was repellent with an MED of 0.043 ± 0.045 mg/cm^2. The last fraction, fraction F, did not show any repellency for the concentration range tested; it contained 1-octen-3-ol. 1-Octen-3-ol is known to exist as two optically active forms. Since the biological activities are likely dependent on the enantiomeric purity of the compounds, we proceeded with characterization of the enantiomers present in fraction F. The (*R*)-(−)-1-octen-3-ol form has a genuine mushroom-like odor that can be used for mushroom aroma authenticity control, whereas (*S*)-(+)-1-octen-3-ol has a moldy, grassy, musty odor.[30] Enantiomerically pure 100% (*R*)-(−)-1-octen-3-ol was determined using chiral separation (Figure 3) and verified by optical rotation {$[\alpha]_D^{25}$ −12.58 (*c* 0.5 in $CHCl_3$)}. It was a weak repellent with an MED of 1.000 ± 0.433 mg/cm^2 (Figures 1 and 2). The other form of 1-octen-3-ol, (*S*)-(+)-1-octen-3-ol, was purchased from Bedoukian Research Co. and tested in repellent bioassays. (*S*)-(+)-1-octen-3-ol exhibited better repellency with an MED of 0.437 ± 0.287 mg/cm^2 compared to that of (*R*)-(−)-1-octen-3-ol (MED 1.000 ± 0.433 mg/cm^2). *M. fistulosa* and *M. bradburiana* essential oils consist of 100% (*R*)-(−)-1-octen-3-ol in both oils. These oils may be considered as the starting point for the isolation and purification of (*R*)-(−)-1-octen-3-ol. However, it was evident that these two compounds were inferior in repellency when compared to the earlier collected fractions, and therefore (unless by a synergistic interaction), (*R*)-(−)-1-octen-3-ol is not likely to contribute significantly to the observed repellent effect of the oil.

A similar type of systematic fractionation procedure was followed for *M. fistulosa* essential oil. Column chromatography of the *M. fistulosa* essential oil resulted in six fractions (Figure 2). No repellent activity was observed in fraction A (rich in *p*-cymene) and weak repellency was detected from fraction B, which had an MED of 0.250 ± 0.109 mg/cm^2. Because of the weak repellency in fraction B, further purification was not performed. Carvacrol methyl ether was obtained from fraction C in pure form, and this fraction showed repellent activity with an MED of 0.063 ± 0.027. Fraction D contained a mixture of 91.71% carvacrol + 8.29% thymol and produced an MED of 0.027 ± 0.018 mg/cm^2. The next fraction, fraction E, contained pure carvacrol and was the most potent repellent fraction with an MED of 0.013 ± 0.009 mg/cm^2. The last fraction F contained pure 100% (*R*)-(−)-1-octen-3-ol (Figures 2 and 3).

All isolated repellent compounds were evaluated as larvicides against 1-day-old *Ae. aegypti* larvae. Thymol was the most toxic compound among tested compounds with a LD_{50} value of 13.9 ppm, followed by carvacrol, eugenol, and carvacrol methyl ether (Table 2). On the basis of LD_{50} values, toxicity in thymol was significantly higher than in carvacrol (20.13 ppm) or eugenol (23.4 ppm). The toxicity of carvacrol methyl ether with an LD_{50} value of 40.6 ppm was significantly lower than the toxicity of the other compounds tested. The LD_{90} values did not differ significantly among thymol, carvacrol, and eugenol. (*R*)-(−)-1-Octen-3-ol did not show any larvicidal activity in the screening bioassays, and its LD_{50} was not calculated.

Although the oils were toxic to mammalian cells, with IC_{50} values in the range of 52−80 μg/mL, the isolated repellent compounds (carvacrol, thymol, eugenol, carvacrol methyl ether, and 1-octen-3-ol) were not individually cytotoxic to any of the cell lines up to a high-test concentration of 20 μg/mL (Table 3). During the bioguided fractionation of oils, we were able to separate the cytotoxic components from the bioactive components, leading to the isolation of compounds with mosquito repellent activity, but without any cytotoxicity. The monoterpene hydrocarbon *p*-cymene constituted a substantial portion of the *Monarda* oils (18% for *M. bradburiana* and 35% for *M. fistulosa*) and may either be responsible for the

Table 3. Cytotoxicity of *M. bradburiana* (Mb) and *M. fistulosa* (Mf) Essential Oils and Pure Compounds from These Oils against a Panel of Six Cell Lines

	IC_{50}[a] (μg/mL)					
Oils/compds	SK-MEL	KB	BT-549	SK-OV-3	Vero	LLC-PK_{11}
M. bradburiana	52	58	57	60	80	58
M. fistulosa	60	60	57	57	75	54
carvacrol	NC[b]	NC	NC	NC	NC	NC
thymol	NC	NC	NC	NC	NC	NC
eugenol	NC	NC	NC	NC	NC	NC
carvacrol methyl ether	NC	NC	NC	NC	NC	NC
(−)-1-octen-3-ol	NC	NC	NC	NC	NC	NC
doxorubicin[c]	0.6	1.3	0.9	1.4	>5	0.65

[a]SK-MEL, human malignant, melanoma; KB, human epidermal carcinoma, oral; BT-549, human ductal carcinoma, breast; SK-OV-3, human ovary carcinoma; Vero, monkey kidney fibroblasts; LLC-PK_{11}, pig kidney epithelial cells. [b]NC = no cytotoxicity. [c]Doxorubicin was a positive standard.

cytotoxicity or potential cytotoxicity of the oils. Another possibility is that a mixture of all constituents or other minor compounds in the oils might be responsible for the cytotoxicity.

Mazza and Marshall[31] reported that the chemical composition of *Monarda* varies depending upon source and cultivar. For example, *M. fistulosa* var. *menthaefolia* from California contained 70% carvacrol, whereas the same species from Manitoba had over 85% geraniol and no carvacrol.[31] They also reported the hybridized *M. fistulosa* var. *menthaefolia* × *M. didyma* contained five chemotypes made up of geraniol, carvacrol, linalool, thymol, and 1,8-cineole and suggested that *Monarda* hybrids may have the potential to be used as commercial sources of geraniol, carvacrol, linalool, thymol, and 1,8-cineole.[31] The same authors also reported that Lawrence found high percentages of thymol and carvacrol in the *M. fistulosa* ssp. *fistulosa* oil.[31] Shanaida reported that thymol (42%) and *p*-cymene (15%) were major constitutes of *M. fistulosa* essential oil.[32] From these results, it can be concluded that *M. fistulosa* essential oil composition and proportions of main compounds vary among the interspecific hybrids and can also be attributed to genotype, as well as geographical and seasonal variations.

Bioassay-guided fractionation of both *Monarda* essential oils revealed that carvacrol (MED of 0.013 ± 0.009 mg/cm^2), thymol (MED of 0.031 ± 0.014 mg/cm^2), eugenol (MED of 0.43 ± 0.045 mg/cm^2), and carvacrol methyl ether (MED of 0.063 ± 0.027 mg/cm^2) exhibited repellency against *Ae. aegypti* in comparison to the standard repellent DEET (MED of 0.039 ± 0.14 mg/cm^2) (Figures 1 and 2). Eugenol has a methylated hydroxyl group and its activity was lower than those carvacrol and thymol. Carvacrol methyl ether, which has a methyl ether group instead of a hydroxyl group, was less active than carvacrol. Park et al. reported that α-terpinene and carvacrol had greater repellency than DEET against *Culex pipiens pallens*, whereas thymol showed the same repellency as DEET.[33] In the present study, carvacrol and thymol showed good activity in both repellent and larvicidal bioassays against *Ae. aegypti*.

Monoterpenoids are widely distributed in essential oils and reported as neurotoxicants against different insect species.[34] Enan assessed the insecticidal activity of five monoterpenoids (*p*-cymene, thymol, carvacrol, α-terpineol, and L-carvone) through the tyramine receptor cascade and found that chemical structure played an important role in toxicity and tyramine receptor binding activity.[35] Tyramine is a neuroactive chemical and has a functional role in the *Drosophila* olfactory system as a neurotransmitter or a neuromodulator.[36] A better knowledge of the molecular mechanisms of action of molecules may lead to a better understanding of insect physiological functions and assist in identification of novel targets for the development of molecularly targeted and environmentally safer pesticides.[35] Carvacrol and thymol derivatives may be good candidates for testing as insect repellents, with active repellent compounds being useful in mode-of-action studies on neurotoxicity and compound-receptor interactions.

The ornamental and traditional medicinal plants *M. bradburiana* and *M. fistulosa* can contribute to native plant gardens, perennial borders, herbal gardens, revegetation plantings, ethnobotanical gardens, and other landscape settings in North America. In addition to the qualities that make these species useful in multiple landscape situations, we also conclude from our study that these species can make further contributions by providing a source of plant-based pest repellents. In addition to their role in attracting insect pollinators to gardens with their showy flowers, essential oils from these American native plant species can also contribute to integrated management of disease-vectoring insects.

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Notes

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